about 50 kD. Thus, the 51-nucleotide stretch common to 31E1 and LK15 is not a cloning artifact. The isolation of WT33 from a pre-B cell cDNA library and the origin of LK15 and 31E1 from separate fetal kidney cDNA libraries suggest that the WIT-2 gene may be alternatively spliced in different tissues.
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31. Tissues were processed for molecular analysis as follows. Whole fetal kidneys and samples of other fetal organs were resected from 12- to 20-week-old fetuses and snap frozen and stored in liquid nitrogen or at −70°C. The 5-year-old and adult transplant donor kidneys, previously perfused with Ringer's lactate solution and kept on ice, were dissected, and slices of cortex were snap frozen in liquid nitrogen and stored. Tumor tissues and adjacent areas of normal kidney were obtained within 1 to 2 hours after nephrectomy, and snap frozen in liquid nitrogen with or without prior embedding in O.C.T. compound (Tissue-Tek, Miles Scientific, Naperoille, IL). Alternatively, tumor tissue was resuspended as a coarse mince in cryoprotectant [10% dimethyl sulfoxide (DMSO), 20 to 50% fetal bovine serum, α-minimum essential medium (MEM)], frozen first at −70°C, and subsequently transferred to liquid nitrogen storage.
32. We thank R. Worton for advice and encouragement; R. Weksberg, A. R. Reeve, and R. J. Wilkins for helpful discussions; R. McInnes and G. Bell for cDNA libraries; and members of the B.R.G.W. laboratory for support and encouragement. This work was supported by a grant from the National Cancer Institute of Canada (NCIC) to B.R.G.W. and H.Y., and a March of Dimes Birth Defects Foundation grant to A.P.F. A.H. was the recipient of a Steve Fonyo NCIC studentship, C.E.C. a Medical Research Council of Canada Scholarship, L.B. an Ontario Graduate Studentship, and M.J.C. an award by the Ter Meulen Fund, The Royal Netherlands Academy of Arts and Sciences.

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Wilms Tumor Locus on 11p13 Defined by Multiple CpG Island-Associated Transcripts

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Wilms tumor is an embryonal kidney tumor involving complex pathology and genetics. The Wilms tumor locus on chromosome 11p13 is defined by the region of overlap of constitutional and tumor-associated deletions. Chromosome walking and yeast artificial chromosome (YAC) cloning were used to clone and map 850 kilobases of DNA. Nine CpG islands, constituting a "CpG island archipelago," were identified, including three islands that were not apparent by conventional pulsed-field mapping, and thus were at least partially methylated. Three distinct transcriptional units were found closely associated with a CpG island within the boundaries of a homozygous DNA deletion in a Wilms tumor.

THE DISTAL HALF OF CHROMOSOMAL band 11p13 has attracted considerable interest as a target for positional cloning because patients with 11p13 deletions develop four abnormalities comprising the WAGR syndrome: Wilms tumor (WT), an embryonal malignancy of the kidney; aniridia, or hypoplasia of the iris; genitourinary dysplasia, including kidney and genital malformations; and mental retardation (*1*). Sporadic, non-WAGR Wilms tumor affects that a gene isolated from 11p13 is the WT gene, by virtue of its location, although its 1 in 10,000 children and represents a putative tumor-suppressor model fulfilling Knudson's two-hit hypothesis, the paradigm of which is retinoblastoma (*2*). However, as we and others have shown, the etiology of Wilms tumor is complex and involves an additional locus at 11p15, and in familial cases a locus on another chromosome (*3*). Two laboratories have proposed expression was unaltered in Wilms tumors (*4, 5*). In this and the accompanying report (*6*), we describe the presence and location of multiple transcribed sequences from this region, including two that show altered expression in some Wilms tumors.

Our starting point in these efforts was S1 (D11S37), a random DNA segment within a region homozygously deleted in WiT-13, a sporadically occurring Wilms tumor (*7*). We had previously set the upper size limit of the WiT-13 deletion at 375 kb, on the basis of mapping of random clones isolated from chromosome- or band-specific libraries (*8*). To define the boundaries of the WiT-13 deletion and to identify regions for more intensive screening for the genitourinary and mental retardation genes, which have been mapped close to WT (*9*), we used yeast artificial chromosome (YAC) cloning, thereby generating a complete physical map of the region, unhampered by DNA methylation of genomic human DNA.

To obtain YACs with human genomic DNA inserts from this region, we synthesized oligonucleotides on the basis of the DNA sequence of S1, and used these as primers to screen a human YAC library (*10*) by polymerase chain reaction (PCR) and filter hybridization (*11*). Southern (DNA) blot hybridization confirmed that two clones, designated yF12 and yG6, included S1 in their sequence, while only yG6 included probe AvH1, which had been isolated by genomic walking and was located 65 kb telomeric from S1. This indicated that the two YACs have only a small region of overlap and thus span a relatively large region of DNA. The YAC clones were mapped by pulsed-field gel electrophoresis (PFGE), by means of partial digestion conditions with a set of eight rare-cutting re-

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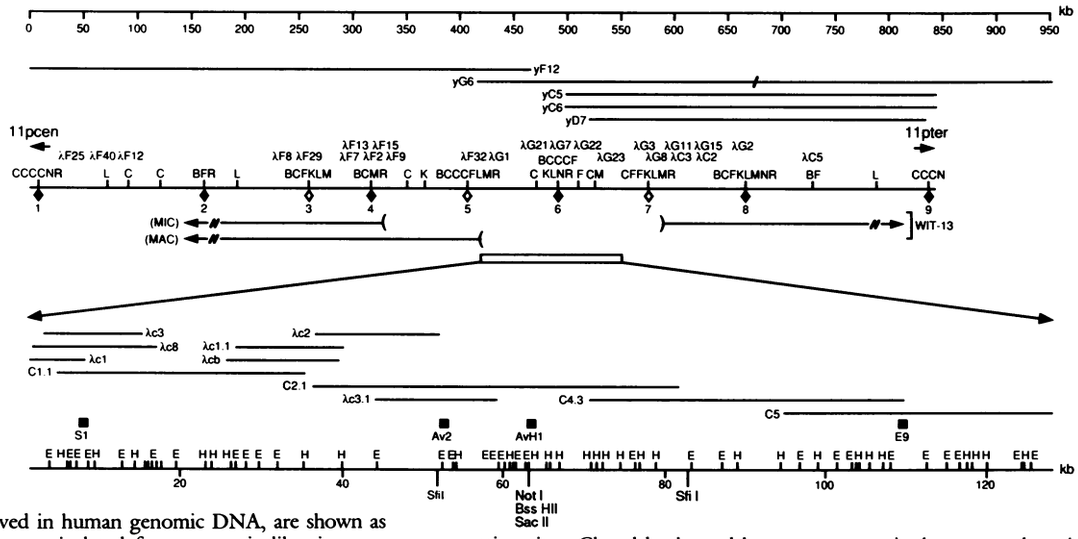

Fig. 1. A physical map of the 11p13 YACs DNA region cloned with YAC vectors and chromosome walking clones. The horizontal lines at the top represent YACs. The slash (/) at the end of clone yG6 indicates junction with a nonsyntenic fragment. The enzymes used were: Bss HII, B; Mlu I, M; Not I, N; Nru I, R; Sac I, C; Sac II, K; Sal I, L; and Sfi I, F. CpG islands, shown as closed diamonds and numbered consecutively, were identified by the coincidence, within the resolving power of PFGE, of three or more rare-cutting restriction enzyme sites. Methylated CpG islands in which most rare-cutting sites were not cleaved in human genomic DNA, are shown as open diamonds. Phage clones were isolated from genomic libraries constructed from each YAC clone (*13*). The chromosomal walk is shown by the open rectangle, and a detailed restriction map of 130 kb was derived from the phage and cosmid clones, shown by horizontal bars at the bottom. Eco RI (E) and Hind III (H) sites are shown above the solid line, as well as some rare-cutting sites. Closed horizontal boxes represent single-copy probes. A schematic representation of WiT-13 chromosome 11 homologs carrying the macroscopic (MAC) and microscopic (MIC) deletions (*7*) is drawn relative to the long-range map. Phage clones are designated by a λ, cosmid clones by c, and YAC clones by y.

striction enzymes, and hybridization with end and internal clones (*12*). Phage libraries were then prepared from each YAC (*13*). To confirm synteny of the clones to chromosome 11, we used a hybrid cell line containing the WiT-13 microdeletion (*7*). We also generated a microcell hybrid cell line containing chromosome 11 (*14*). This analysis revealed a region of nonsynteny in the most distal 250 kb of yG6, presumably due to a co-cloning event during library construction. Subsequently, oligonucleotides were derived from AvH1 and used as PCR primers for identification of additional YAC clones. Four additional YACs—yC5, yA12, yC6, and yD7—were obtained. All but yA12 showed identical pulse-field maps as well as synteny to chromosome 11, allowing us to extend the map of this region to850 kb. Using this map and the phageclones derived from the YACs, we defined and mapped the homozygous deletion of WiT-13 to 175 kb, considerably smaller than the previous estimate (*8*) (Fig. 1).

Our map included rare-cutting restriction endonuclease sites previously identified by direct pulsed-field mapping of human DNA (*4, 5, 7–9*). However, there were a large number of sites apparent only in the YACs. These represent methylated (and transparent) sites in human genomic DNA; as rare-cutting enzymes are inhibited by genomic DNA methylation, whereas the YACs are unmethylated when propagated in yeast. Bird *et al.* (*15*) have described 1- to 10-kb CpG-rich "islands," in which CpG dinucleotides are abundant and unmethylated, in the vicinity of housekeeping genes. Coinci-

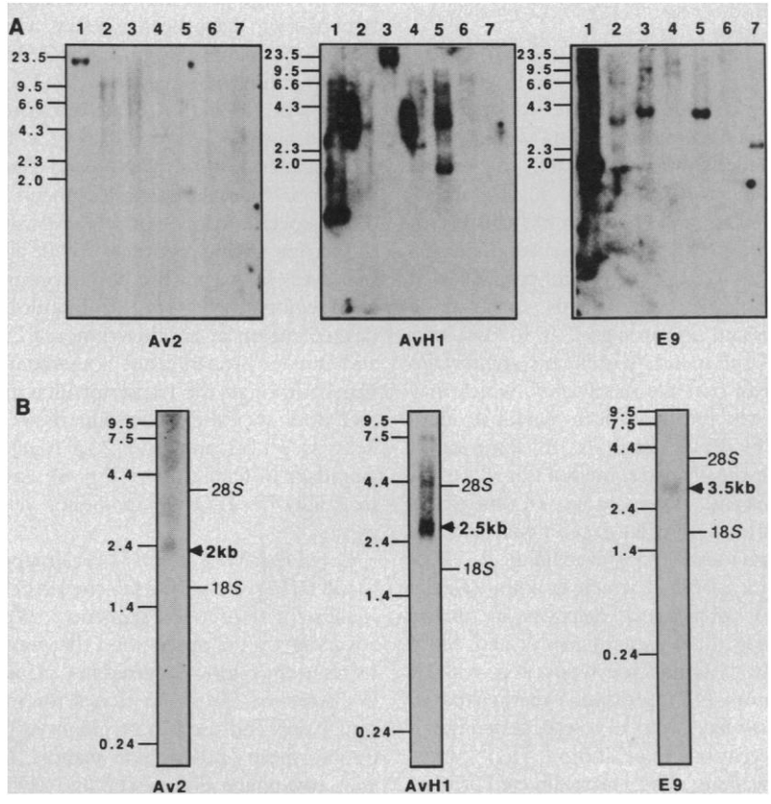

Fig. 2. Detection of conserved DNA probes that contain sequences that are expressed in human kidney. (**A**) Genomic DNA (10 μg per lane) from (lane 1) human; (lane 2) mouse; (lane 3) hamster; (lane 4) rat; (lane 5) cow; (lane 6) dog; and (lane 7) chicken was digested with Hind III. After electrophoresis and blotting onto GeneScreen Plus membranes (New England Nuclear), the filters were hybridized at 42°C in 30% formamide, 5× saline sodium citrate (SSC), 1× Denhardt's solution, with DNA probes radioactively labeled as in Fig. 1. The most stringent wash was at 60°C in 0.2× SSC and 0.1% SDS for 30 min. The probes used for hybridization are indicated. (**B**) Total RNA (10 μg), for probes Av2 and E9, or poly(A)$^+$ RNA (2 μg), for probe AvH1, prepared from adult human kidney (*20*), were separated on a 1% agarose-formaldehyde gel. After blotting onto GeneScreen Plus membranes, hybridizations were performed at 65°C in 1% bovine serum albumin (BSA), 0.5 M sodium phosphate, 1 mM EDTA, and 7% SDS. The filters were washed as in Fig. 1. The arrows indicate the position of the transcripts. The positions of the 28*S* and 18*S* ribosomal RNA bands are also shown.

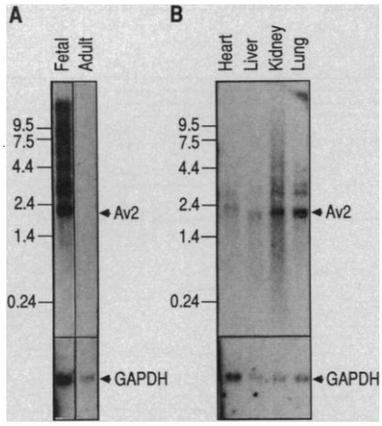

Fig. 3. Tissue distribution and developmental expression of Av2 transcript. Polyadenylated RNA (1 μg) isolated from (**A**) human fetal and adult kidney, and (**B**) human fetal heart, lung, kidney, and liver, was separated by electrophoresis on a 1% agarose-formaldehyde gel, transferred onto GeneScreen membranes, and hybridized with probe Av2 (Fig. 1). RNA isolation and Northern hybridization were performed as in Fig. 2B. The position of hybridizing bands in relation to RNA markers is indicated. Rehybridization to glyceraldehyde phosphate dehydrogenase (GAPDH) (*21*) was used to monitor RNA loading.

dent rare-cutting sites identified by PFGE are assumed to represent CpG islands (*4, 5, 8, 9*), an idea confirmed by DNA sequencing in the case of island 6 (Fig. 1). The YAC-derived map revealed multiple clusters of rare-cutting sites, at roughly 100-kb intervals (Fig. 1). We therefore propose that these multiple CpG islands constitute a "CpG island archipelago." It follows that some of the islands within this archipelago are at least partially methylated, which may explain why they have been previously inapparent by PFGE analysis. In some cases, methylation affected some but not all sites in a CpG island. For example, we observed a Not I site at position 670 kb that had not been previously recognized (Fig. 1). Furthermore, island 7, which is within all the reported homozygous deletions in tumors (*4, 5*) (Fig. 1) is partially methylated. Since methylated islands are associated with X inactivation (*16*), autosomal methylated islands may have a novel role in gene regulation or genomic imprinting.

Our walking efforts were directed primarily toward cloning the DNA sequences flanking CpG island 6, since CpG islands are associated with transcribed genes (*15*), and island 6 is localized within the center of the WiT-13 homozygous deletion. The bidirectional walk resulted in the cloning of a region of 130 kb (Fig. 1) (*17*). Four recombinant clones that included S1 were unstable at their telomeric ends on propagation in bacteria. However, the YAC yF12 appeared stable in this region, and clone λF32, which defines the WiT-13 macrodeletion breakpoint, delimited this region to 9 kb. The chromosomal rearrangement representing one of the WiT-13 deletion breakpoints is localized precisely within this 9-kb segment. The chromosomal walk included all but 45 kb of the DNA within the WiT-13 homozygous deletion as mapped by the YACs (Fig. 1).

Based on the prediction that DNA fragments containing coding exons would be conserved among different species, single-copy probes were used to screen for phylogenetically conserved sequences (Fig. 2A). Three conserved sequences (Av2, AvH1, and E9) were identified, all of which recognized transcripts expressed in human fetal kidney (Fig. 2B). Probe Av2 was located 9 kb centromeric from island 6, and detected a transcript of 2 kb. Probe AvH1, which mapped to and included island 6, identified a 2.5-kb transcript. Probe E9 mapped 35 kb toward the telomere from island 6 and detected a 3.5-kb transcript. Human kidney cDNA libraries were screened with all three probes. Complementary DNA clones for two of them have been isolated: GB16, a 2-kb cDNA clone recognized by AvH1 and corresponding to the gene WIT-1; and 31E1, a 2.7-kb cDNA clone recognized by E9 and corresponding to the gene WIT-2 (*6*). Isolation of these cDNAs and characterization of their tissue-, developmental-, and tumor-specific expression are described in the accompanying report (*6*). While all three sequences lie within the homozygously deleted region of WiT-13, Av2 lies outside the deleted region of another tumor, PER (*5*), and thus is probably not a candidate WT gene. Although the transcript identified by Av2 does not exhibit the same tissue specificity as WIT-1 and WIT-2, it is relatively abundant in fetal kidney (Fig. 3), and thus may also be involved in kidney development.

Given that WIT-1 and WIT-2 mapped to island 6 (Fig. 1) (*6*), we screened other CpG islands for transcribed sequences. We took advantage of the observation that most Nru I sites in the region occurred in CpG islands. We screened 50 phage clones for internal Nru I sites and used those clones as probes for Northern (RNA) blot analysis. In this way, two phage clones, λF2 and λF7, were found to detect transcripts of sizes 2.0 and 3.7 kb, respectively, in human fetal kidney (*18*). Although λF2 and λF7 are not deleted in WiT-13 tumor DNA, the regions to which these transcribed sequences map may be relevant to genitourinary dysplasia (*9*). No transcripts have yet been identified with clones from the region of island 7, which does map within the WiT-13 deletion.

These data have three important implications. First, we have identified a large number of genes expressed in fetal kidney and located within the WAGR region. Mutations in any one of these genes may be involved in WT or genitourinary dysplasia. Second, we have cloned an 850-kb region in YAC and phage vectors. It will be relatively straightforward to clone other genes in the WAGR complex by isolating adjacent YAC clones. Furthermore, since chromosome 11 confers a tumor suppressor phenotype in WT (*19*), the YACs can be used directly in genetic complementation experiments, an important advantage given the identification of multiple candidate genes. Third, we have identified three CpG islands in this region that are at least partially methylated in DNA from cultured lymphoblasts and fibroblasts, the cells used in conventional genomic pulsed-field mapping. The cloning of these islands will now permit direct examination of their methylation pattern in diverse tissue types. We hypothesize that several genes important in normal kidney development, which may be involved in WT or genitourinary dysplasia, lie within a CpG island archipelago. Our observation of methylation of some of these islands suggests a potential molecular basis for either normal developmental regulation or genomic imprinting of these genes.

Note added in proof: We have recently observed that the left end of YAC yF12, outside the region of the WiT-13 deletion and transcripts reported here, although syntenic to some chromosome 11 hybrids, is nonsyntenic to others and thus may not be contiguous to the remainder of the YAC.

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12. YAC blocks were prepared as described (*10*). Partial digestions were controlled by variation of enzyme concentration for a fixed time of 1.5 hours. Field-inversion gel electrophoresis was performed with a DNAStar Pulse electrophoresis programmer, at 220 V for 36 hours at 4°C, with a 9 to 120s ramp and a ratio of 3:1. Molecular weight standards were lambda phage concatamers (Beckman) and *Saccharomyces cerevisiae* strain CF22 prepared in blocks. Gels were exposed to 254 nm ultraviolet light for 30 s, and then transferred under alkaline conditions to GeneScreen filters. Labeled probes were prepared by the method of A. P. Feinberg and B. Vogelstein [*Anal. Biochem.* **132**, 6 (1983)], and hybridization was performed by the method of G. M. Church and W. Gilbert [*Proc. Natl. Acad. Sci. U.S.A.* **18**, 1991 (1984)].
13. High molecular weight yeast DNA was prepared

and purified from sucrose gradients as described (*10*). Partial Mbo I digests were size-fractionated by PFGE, and 9- to 25-kb DNA was ligated into the Bam HI site of λEMBL3 (StrataGene), and packaged with Gigapak Gold (StrataGene) and plated on *Escherichia coli* P2392. Phage plaques containing human DNA inserts were identified by their ability to hybridize to L1 [L. B. Kole, *J. Mol. Biol.* **165**, 257 (1983)] and Alu [W. R. Jelinek *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 77, 6130 (1980)] repeats and total human DNA.

14. Mouse A9 cells containing a single human chromosome with a translocation between chromosome 11 and a small portion of the X chromosome (Xqter → Xq26:11q23 → 11pter) were constructed by the microcell transfer technique [R. E. K. Fournier and F. H. Ruddle, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 319 (1977); M. Koi *et al.*, *Jpn. J. Cancer Res.* (GANN) **80**, 122 (1989)]. The microcell donor cells were the hybrids formed between human fibroblast GM 03552 (Human Genetic Mutant Cell Repository, Camden, NJ) and hypoxanthine-guanine phosphoribosyltransferase (HPRT)–deficient A9 cells. The hybrids contain several human chromosomes including chromosome 11. A9 cells were again used as recipient cells for a microcell transfer, and cells with a transferred chromosome X/11 containing the *HPRT* gene were selected in medium containing hypoxanthine, aminopterin, and thymidine (HAT).
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17. Isolation of probe S1 and cosmid clone C1.1 has been described (7). Two genomic DNA phage libraries were used: one was obtained from J. M. Rommens [J. M. Rommens *et al.*, [*Science* **245**, 1059 (1989)], and the other, constructed with Mbo I partially cut human DNA cloned into λEMBL3 vector, was provided by N. Jordan. Two cosmid libraries with vectors pEW15 [G. M. Wahl *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 242 (1987)] and sCos-1 [G. A. Evans *et al.*, *Gene* **79**, 9 (1989)], to clone partially digested (Sau3A) human lymphoblast DNA, were provided by C. McDowell.
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22. We thank R. Worton and D. Schlessinger for encouragement and advice; L.-C. Tsui, R. Weksberg, M. Minden, C. Thompson, J. Lowe, M. Tewari, and F. Collins for helpful discussions; S. Bodrug, C. McDowell, N. Jordan, J. Rommens, and M. Olson, for providing genomic libraries; C. Campbell and M. S. McAndrews-Hill for sequencing; M. Glaves for technical assistance; and A. Huntzicker for preparing the manuscript. This work was supported by Clinical Research grant no. 6-551 from the March of Dimes Birth Defects Foundation grant (A.P.F.); the NCIC (B.R.G.W. and H.Y.); a Steve Fonyo NCIC studentship (A.H.); an Ontario Graduate studentship (L.B.); the New Zealand Cancer Society (A.E.R.); and NIH grants CA48932 (A.P.F.) and T32DK07458 (S.E.K.) and T32GM07315 (L.M.K.).

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Regulation of Gene Expression with Double-Stranded Phosphorothioate Oligonucleotides

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Alteration of gene transcription by inhibition of specific transcriptional regulatory proteins is necessary for determining how these factors participate in cellular differentiation. The functions of these proteins can be antagonized by several methods, each with specific limitations. Inhibition of sequence-specific DNA-binding proteins was achieved with double-stranded (ds) phosphorothioate oligonucleotides that contained octamer or κB consensus sequences. The phosphorothioate oligonucleotides specifically bound either octamer transcription factor or nuclear factor (NF)-κB. The modified oligonucleotides accumulated in cells more effectively than standard ds oligonucleotides and modulated gene expression in a specific manner. Octamer-dependent activation of a reporter plasmid or NF-κB–dependent activation of the human immunodeficiency virus (HIV) enhancer was inhibited when the appropriate phosphorothioate oligonucleotide was added to a transiently transfected B cell line. Addition of phosphorothioate oligonucleotides that contained the octamer consensus to Jurkat T leukemia cells inhibited interleukin-2 (IL-2) secretion to a degree similar to that observed with a mutated octamer site in the IL-2 enhancer. The ds phosphorothioate oligonucleotides probably compete for binding of specific transcription factors and may provide anti-viral, immunosuppressive, or other therapeutic effects.

ANALYSIS OF THE FUNCTION OF EUkaryotic transcriptional regulatory proteins in mammalian cells has been limited. Because such transcription factors are often essential to cell viability, mutant cell lines lacking these proteins can be difficult to obtain. An alternative approach is to generate trans-dominant mutants that interfere with the function of transactivators. While this strategy has been successful (*1*), the generation of such mutants is not always possible. Another method utilizes promoter competition, whereby plasmids containing cis-acting elements in common with an indicator gene are introduced in high copy number into cells (*2*). Because these plasmids must be maintained uniformly in large numbers of cells, this approach has also been limiting. Advances in the synthesis of DNA now allows an alternative approach to this problem. Oligonucleotides with modified phosphodiester bonds, such as phosphorothioate, methyl phosphate, phosphoramidite, or methyl phosphonate derivatives can be routinely synthesized in large amounts and are relatively resistant to nucleases (*3*, *4*). Because of their increased cell permeability and stability, these compounds have been used as antisense agents (*5*). We sought to ascertain whether double-stranded (ds) phosphorothioates could penetrate cells, bind sequence-specific DNA-binding proteins, and interfere with eukaryotic transcription in vivo.

To determine whether ds phosphorothioates could compete for binding of sequence-specific DNA-binding proteins, we used ^{32}P-labeled ds oligonucleotides that contained the octamer or κB elements in the electrophoretic mobility shift assay (EMSA). An octamer site derived from the IL-2 enhancer and nuclear extracts from Jurkat cells were used for these studies (*6*–*8*). Protein binding to the ^{32}P-labeled IL-2 octamer probe (unmodified) was inhibited equally well by unlabeled ds oligonucleotide or phosphorothioate octamer sequences, but not by κB sequences of either type (Fig. 1A). Conversely, protein binding to ^{32}P-labeled κB probe (unmodified) was inhibited in an indistinguishable manner by ds oligonucleotide or phophorothioate κB sequences, but not by octamer sequences of either type (Fig. 1B). When phosphorothioates were used as labeled probes, we observed specific binding using octamer (Fig. 1C) or κB (Fig. 1D) in the EMSA. In addition, protein-binding to the phosphorothioates was inhibited equally well by the appropriate unmodified or phosphorothioate oligonucleotides (Fig. 1), but not by oligonucleotides with mutations within the appropriate consensus binding site (*9*). Because phosphorothioates competed with equimolar potency, they may serve as potential antagonists, despite the fact that they differ chemically from standard DNA and may not interact with DNA binding proteins in an identical manner. The phosphorothioates also showed increased resistance to digestion by deoxyribonuclease (DNase) I (*9*), as described for other endonucleases (*10*).

To compare the efficacy of cellular incorporation of ds phosphorothioate and phosphodiester oligonucleotides, we incubated ^{32}P-labeled samples of each type (octamer sequences) with Jurkat T leukemia cells.

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